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INIT 87/26/94

MORPHOLOGICAL TRANSFORMATION ASSAY  
USING BALB/3T3 MOUSE EMBRYO CELLS

TEST ARTICLES  
9 ZINC DIALKYLDITHIOPHOSPHATES  
ZINC CHLORIDE  
CALCIUM DIALKYLDITHIOPHOSPHATE

FINAL REPORT

FOR

CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M. STREET, N.W.  
WASHINGTON, D.C. 20037

BY

MICROBIOLOGICAL ASSOCIATES  
5221 RIVER ROAD  
BETHESDA, MARYLAND 20816

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ASSOCIATES**

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## INTRODUCTION

The BALB/3T3 transformation assay was designed to allow the expression of transformed foci of high cell density and aberrant cell morphology on a confluent monolayer of nontransformed, contact-inhibited 3T3 cells. This system has been demonstrated to be sensitive to the transforming activity of a variety of chemicals (1,3).

The BALB/3T3 Clone A31-1 cell line was derived by Dr. T. Kakunaga, National Cancer Institute, Bethesda, MD from the aneuploid, nontumorigenic BALB/3T3 Clone A31 cell line (1). This subclone is characterized by postconfluence inhibition of proliferation and the ability to form distinct quantifiable foci of aberrant cell morphology following exposure to chemical carcinogens.

The non-activated assay was performed by exposing BALB/3T3 cells, in monolayer, to three concentrations of the test article as well as positive and negative controls for 24 hours in the absence of a supplemental exogenous mammalian metabolic activation system, after which the cells were cultured for estimation of the cytotoxic effects of treatment and the induction of phenotypic transformation (3).

If a negative result was obtained and/or upon authorization by the Sponsor, an activation assay was performed by exposing BALB/3T3 cells, in suspension, to three to five concentrations of the test article as well as positive and negative controls for 2 hours in the presence of an S-9 activation system, after which the cells were cultured for estimation of the cytotoxic effects of treatment and the induction of phenotypic transformation (3).

If a negative result was obtained and upon authorization of the Sponsor, an activation assay was performed by exposing BALB/3T3 cells, in monolayer, to three concentrations of the test article as well as positive and negative controls for 4 hours in the presence of an S-9 activation system, after which the cells were cultured for estimation of the cytotoxic effects of treatment and the induction of phenotypic transformation (3).

## PURPOSE

The purpose of this study is to assess the morphological transforming potential of the test article in the BALB/3T3 mouse embryo cell line.

## MATERIALS AND METHODS

### Materials

**Mammalian Cells:** BALB/3T3 Clone A31-1 mouse embryo cells (Dr. T. Kakunaga, National Cancer Institute, Bethesda, Maryland).

### **Biological Reagents:**

Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin-streptomycin (complete EMEM)

Hanks' balanced salt solution (HBSS)

HBSS, Calcium and Magnesium-free (CMF-HBSS)

Dulbecco's phosphate buffered saline (PBS)

Trypsin, 0.25% in CMF-HBSS

Methanol, 95%

Giemsa stain

Cofactor Pool: 3.8 mM nicotinamide adenine

dinucleotide phosphate, tetrasodium salt,

Type III (NADPH), 3.8 mM nicotinamide

adenine dinucleotide phosphate, sodium salt

(NADP), 2.8 mM nicotinamide adenine

dinucleotide phosphate, disodium salt,

Grade III (NADH), 14.8 mM glucose-6-

phosphate in Dulbecco's PBS, pH 7.6

S-9, 9000 x g supernatant of Aroclor 1254-

induced Fischer 344 rat liver homogenate

### **Supplies:**

Glass tubes with screw caps

Conical centrifuge tubes with caps, 50 cc

Pipets, assorted sizes

Plastic tissue culture flasks and dishes

### **Chemicals:**

Solvent for test article

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

Benzo(a)pyrene (BaP)

## Methods

The S-9 was prepared according to established procedures. Adult male Fischer 344 rats, 200-250 gm, were induced by a single intraperitoneal injection of Aroclor 1254 at a dose of 500 mg/kg body weight two days prior to sacrifice. The animals were sacrificed and the livers aseptically removed. The excised tissue was rinsed three times in cold sterile 0.15M KCl and then homogenized in a Polytron Tissuemizer at a concentration of 1:3 w/v in 0.15M KCl. The supernatant fraction (S-9) was collected following centrifugation in the cold at 9000 x g for 20 min, portioned into aliquots for daily use, and stored frozen at -70°C until used. Each batch of S-9 was characterized by its ability to metabolize 2-aminoanthracene and benzo(a)pyrene to forms mutagenic to S. typhimurium.

For the assay without exogenous metabolic activation, exponentially growing 3T3 clone A31-1 cells were seeded for each treatment condition at 250 cells/60 mm dish in triplicate for determination of cytotoxicity and at  $1 \times 10^4$  cells/60 mm dish in 12-15 replicates for determination of phenotypic transformation and were incubated at 36°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 20-24 hours.

The time of initiation of chemical treatment was designated as day 0. Concentrations of the test article were prepared immediately prior to use. The test article was dissolved in the appropriate solvent, after which working dilutions (2X final concentrations) were prepared in complete EMEM. Cells were exposed to three concentrations of test article as well as solvent and positive controls for 24 hours at 36°C. Treatment was carried out by adding 2X concentrations of test article, solvent, or positive control to an equal volume of complete EMEM in the dish. Following the exposure period, all treatment media were withdrawn, the cells were washed once with HBSS and refed with 5 ml complete growth medium. Using a permanent marking pen, cytotoxicity and transformation plates were identified by the study number and a code system to designate the treatment condition, test phase, and replicate plate number.

After 7-10 days incubation, the concurrent cytotoxicity dishes were fixed with methanol, stained with 10% aqueous Giemsa, and scored for colony formation. After 4-6 weeks incubation with twice weekly medium changes, the transformation dishes were fixed with methanol, stained with 10% aqueous Giemsa, and scored for morphologically transformed Type II and Type III foci according to the criteria established by Reznikoff et al. (2).

For the assay in the presence of exogenous metabolic activation, exponentially growing BALB/3T3 clone A31-1 cells were suspended in copical treatment tubes at a density of approximately  $2 \times 10^6$  cells in 2 ml PBS containing the S-9 reaction mixture. Two ml of the test article, positive control

or solvent control dosing solution (2X final concentration) were added to the cell suspension. The resulting treatment medium contained approximately  $2 \times 10^6$  cells in 4 ml PBS containing 100  $\mu$ l S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. Treatment was carried out on a rocking platform at 36°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 2 hours.

Using a permanent marking pen, the treatment vessels were identified with the study number, treatment and dose level. Cytotoxicity and transformation plates were identified by the study number and a code system to designate the treatment condition, test phase, and replicate plate number.

For evaluation of cytotoxicity, cells from each treatment condition were seeded in complete medium, in triplicate, at a density of 250 cells/60 mm dish. After 7-10 days incubation at 36°C, the colonies were fixed with methanol, stained with 10% aqueous Giemsa, and counted.

For expression of the transformed phenotype, cells from each treatment condition were seeded in complete medium, 12-15 dishes per treatment, at a density of approximately  $10^4$  cells/60 mm dish. After 4-6 weeks incubation at 36°C with twice weekly medium changes, the transformation plates were fixed with 95% methanol, stained with 10% aqueous Giemsa, and scored for morphologically transformed Type II and Type III foci according to the criteria established by Reznikoff et al. (3).

#### Controls

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, CAS 70-25-7), 97% purity, lot 111247, was obtained from Aldrich Chemical Company, Milwaukee, WI and was used as the positive control in the non-activated study. Benzo(a)pyrene (BaP, CAS 50-32-8), practical grade, lots 61C-1350 and 102F-0319, was obtained from Sigma Chemical Company, Saint Louis, MO and was used as the positive control in the activation study.

#### Evaluation of Test Results

The cytotoxic effects of each treatment condition are expressed relative to the solvent-treated control (relative cloning efficiency). The transformation frequency for each treatment condition is expressed as the number of transformed foci per surviving cell. The confidence level associated with the number of surviving cells employed in the selection process requires that the assumption be made that were more cells utilized for the selection of transformants, the possibility exists that at least one Type III focus could be obtained. Therefore, for test conditions in which no Type III foci were observed, transformation frequencies are expressed as less than the frequency obtained with one Type III focus. The number of

Type II and Type III foci per total dishes scored are also presented.

The transforming potential of each treatment condition was compared to that of the solvent control using a special application of the Poisson distribution as follows:

The proportion (P) of all cells not subject to induced transformation is equal to the number of surviving cells in the solvent control group divided by the total of the surviving cells in the solvent control and treatment group. Significance is determined by:

$$p(X \geq n_1/n) = \frac{n!}{n_1! (n-n_1)!} P^{n_1} (1-P)^{n-n_1}$$

where p is the probability, n is the number of induced Type III foci,  $n_1$  is the number of spontaneous Type III foci, and P is defined above. If p is very small ( $p < 0.05$ ) the hypothesis that the probability of a transformation event is the same for both the treated and control groups is rejected and the induced transformation frequency is considered significant at the  $p < 0.05$  level.

#### Criteria for Determination of a Valid Test

The cloning efficiency of the solvent control must be equal to or greater than 25%. The relative survival of cells exposed to the test article must fall within 30-60% for one dose level and 60-90% for another dose level. This requirement may be waived in the event of a positive result, or if solubility limits preclude testing in a toxic range. The number of Type III foci in the negative control must not exceed 1 focus per total replicate dishes. The positive control must induce a significant ( $p < 0.05$ ) number of Type III foci relative to the negative control.

## SUMMARY

Nine coded zinc dialkyldithiophosphates, zinc chloride and calcium dialkyldithiophosphate were tested in the BALB/3T3 morphological transformation assay. Where possible, dose levels were selected to yield a relative survival of 30-60% and 60-90% for two of the three to five dose levels tested. All nine zinc dialkyldithiophosphates were tested by exposure for 24 hours in the absence of an exogenous mammalian metabolic activation system. Those found negative in the non-activated assay were retested by exposing cells, in suspension for 2 hours, in the presence of an aroclor-induced Fischer rat S-9 activation system. Based upon the findings of these studies, the test articles could be placed into two general categories: highly suspect or positive. CMA-107, CMA-108 and CMA-109 were concluded to be highly suspect because the induced transformation frequencies were either statistically significant at highly toxic doses only or were not statistically significant but substantially increased at moderate to non-toxic dose levels. CMA-102, CMA-105, CMA-106, CMA-110, CMA-111 and CMA-112 were positive because the induced transformation frequencies were either statistically significant at moderate to non-toxic dose levels or were statistically significant at highly toxic doses with substantial non-statistical increases at moderate to non-toxic dose levels. For CMA-102, CMA-106, CMA-108 and CMA-110, activity was found only in the presence of an exogenous source of metabolic activation. Zinc chloride and calcium dialkyldithiophosphate were tested in the S-9 activated assay only, with zinc chloride being positive and calcium dialkyldithiophosphate negative.

CMA-102



BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-102)

Test Article Lot No.: Not Provided

MA Study No.: T1856.302002

Test Article Description: Clear Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 11/24/82

Initiation Date: 11/24/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor:

CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility:

MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director:

Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician:

John T. McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article CMA-102 was received by Microbiological Associates on 11-24-82 and was assigned the code number T1856. Storage requirements and expiration date were not specified. The test article was stored at room temperature. The test article was dissolved in acetone (CAS 67-64-1), lots 132200 and 110844, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. At the time of testing, T1856 was characterized as a clear viscous liquid.

T1856 was tested in the absence of an exogenous metabolic activation system at doses of 30, 15 and 8 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 30, 15 and 8 ug/ml in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 49%, 66% and 95% at 30, 15 and 8 ug/ml, respectively. The activity of T1856 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 2. One spontaneous Type III focus was observed in the solvent control for a background transformation frequency of  $0.12 \times 10^{-4}$ . No foci were observed in the test article-treated groups. MNNG induced 6 Type II and 17 Type III foci for a transformation frequency of  $28.33 \times 10^{-4}$  ( $p < 0.01$ , Modified Poisson Distribution).

T1856 was tested in the presence of an S-9 activation system at doses of 6, 5 and 4 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. A repeat assay was performed at 8, 7 and 6 ug/ml in an effort to obtain higher levels of toxicity. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with PBS. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 2 hour treatment of BALB/3T3 clone A31-1 cells with 8, 7, 6, 5 and 4 ug/ml in the presence of an exogenous metabolic activation system are presented in Table 3. Relative to the acetone control, the cell survival was approximately 65%, 79% and 95% at 6, 5 and 4 ug/ml, respectively (Experiment 1) and 0%, 0% and 55% at 8, 7 and 6 ug/ml, respectively (Experiment 2). The activity of T1856 in the transformation system after a 2 hour treatment in the presence of an S-9 activation system is presented in Table 4. No spontaneous Type III foci were observed in the solvent control for background transformation frequencies of  $< 0.17 \times 10^{-4}$  in Experiment 1 and  $< 0.15 \times 10^{-4}$  in Experiment 2. In Experiment 1, two Type II and one Type III foci were observed at 6 ug/ml, five

( Type II and four Type III were observed at 5 ug/ml, and one Type II and three Type III foci were observed at 4 ug/ml. The induced transformation frequency of  $0.78 \times 10^{-4}$  was statistically increased ( $p < 0.05$ , Modified Poisson Distribution) relative to the acetone control. In Experiment 2, 8 and 7 ug/ml were toxic. Two Type III foci were observed at 6 ug/ml. BaP induced 9 Type II and 6 Type III foci (Experiment 1) and 6 Type II and 7 Type III foci (Experiment 2) for transformation frequencies of  $2.4 \times 10^{-4}$  and  $3.3 \times 10^{-4}$ , respectively ( $p < 0.01$ , Modified Poisson Distribution).

### CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicate that the test article T1856 (CMA-102) does induce morphological transformation in BALB/3T3 clone A31-1 cells in the presence of an exogenous source of metabolic activation.

TABLE 1

CYTOTOXIC EFFECTS OF T1856 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.55	100.0
MNNG 0.5 ug/ml	0.04	7.3
T1856 30 ug/ml	0.27	49.1
15 ug/ml	0.36	65.5
8 ug/ml	0.52	94.5

<sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.

<sup>b</sup> Cloning efficiency is reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency is reported as the number of colonies per treatment condition relative to 100% survival in the solvent control.

TABLE 2

TRANSFORMATION POTENTIAL OF T1856 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> ( $\times 10^{-4}$ )
	Type II	Type III	
Acetone 2 ul/ml	0/15	1/15	0.12
MNNG 0.5 ug/ml	6/15	17/15	28.33 <sup>c</sup>
T1856 30 ug/ml	0/15	0/15	<0.25
15 ug/ml	0/14	0/14	<0.20
8 ug/ml	0/14	0/14	<0.14

<sup>a</sup> Cells seeded at  $10^4$  cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup>  $p < 0.01$ , Modified Poisson Distribution

TABLE 3

CYTOTOXIC EFFECTS OF T1856 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE PRESENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
EXPERIMENT 1		
Acetone 2 ul/ml	0.43	100.0
BaP 12.5 ug/ml	0.18	41.9
T1856 6 ug/ml	0.28	65.1
5 ug/ml	0.34	79.1
4 ug/ml	0.41	95.3
EXPERIMENT 2		
Acetone 2 ul/ml	0.44	100.0
BaP 12.5 ug/ml	0.14	31.8
T1856 8 ug/ml	0.00	0.0
7 ug/ml	0.00	0.0
6 ug/ml	0.24	54.5

<sup>a</sup> Cells were treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml treatment medium containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NadH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of 250 cells/60 mm dish. After 7-10 days incubation, the cells were fixed, stained and scored for colony formation.

<sup>b</sup> Cloning efficiency is reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

TABLE 4

TRANSFORMATION POTENTIAL OF T1856 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
EXPERIMENT 1			
Acetone			
2 ul/ml	0/14	0/14	<0.17
BaP			
12.5 ug/ml	9/14	6/14	2.38 <sup>c</sup>
T1856			
6 ug/ml	2/15	1/14	0.24 <sup>d</sup>
5 ug/ml	5/15	4/15	0.78 <sup>d</sup>
4 ug/ml	1/15	3/15	0.49
EXPERIMENT 2			
Acetone			
2 ul/ml	1/15	0/15	<0.15
BaP			
12.5 ug/ml	6/15	7/15	3.33 <sup>c</sup>
T1856			
8 ug/ml	- TOXIC -		
7 ug/ml	- TOXIC -		
6 ug/ml	0/15	2/15	0.56

<sup>a</sup> Cells were treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml treatment medium containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of  $10^4$  cells/60 mm dish. After 4-6 weeks incubation, the cells were fixed, stained and scored for morphological transformation.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup>  $p < 0.01$ , Modified Poisson Distribution

<sup>d</sup>  $p < 0.05$ , Modified Poisson Distribution

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY

Study Number: T1856.302002

Study Director: D. PUTMAN, PH.D.

Initiation Date: NOVEMBER 24, 1982

( Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.



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DATES/PHASES

( INSPECT ON 82/11/29 - 82/11/29, TO STUDY DIR 82/11/29, TO MGMT 82/11/29

PHASES: PROTOCOL REVIEW

INSPECT ON 83/02/04 - 83/02/04, TO STUDY DIR 83/02/04, TO MGMT 83/02/07

PHASES: SCORING TRANSFORMATION PLATES

INSPECT ON 83/04/14 - 83/04/14, TO STUDY DIR 83/04/14, TO MGMT 83/04/18

PHASES: TREATMENT OF TEST SYSTEM WITH TEST AND CONTROL ARTICLES

INSPECT ON 83/05/19 - 83/05/19, TO STUDY DIR 83/05/19, TO MGMT 83/05/28

PHASES: TREATMENT OF TEST SYSTEM WITH TEST ARTICLE AND POSITIVE CONTROL

INSPECT ON 83/08/15 - 83/08/16, TO STUDY DIR 83/08/16, TO MGMT 83/10/27

PHASES: DRAFT REPORT  
(

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

*Nona S. Karter*

Quality Assurance  
RA/QA Department

*9/25/84*  
Date

CMA-105

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-105)

Test Article Lot No.: Not Provided

MA Study No.: T1866.302002

Test Article Description: Pale Yellow, Viscous liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 12/10/82

Initiation Date: 12/21/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor: CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director: Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician: John T. McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article CMA-105 was received by Microbiological Associates on 12-10-82 and was assigned the code number T1866. Storage requirements and expiration date were not specified. The test article was stored at room temperature. The test article was dissolved in acetone (CAS 67-64-1), lot 110844, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. at the time of testing, T1866 was characterized as a pale yellow, viscous liquid.

T1866 was tested in the absence of an exogenous metabolic activation system at doses of 40, 30, and 20 mg/ul which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 40, 30 and 20 ug/ml T1866 in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 2%, 43%, and 83% at 40, 30 and 20 ug/ml, respectively. The activity of T1866 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 2. One spontaneous Type III focus was observed in the solvent control for a background transformation frequency of  $0.13 \times 10^{-4}$ . three Type II and two Type III foci were observed at 40 ug/ml, five Type II and four Type III foci were observed at 30 ug/ml, and one Type III focus was observed at 20 ug/ml. The transformation frequency of  $14.29 \times 10^{-4}$  at 40 ug/ml was statistically increased ( $p < 0.05$ ) when compared to that of the acetone control. MNNG induced three Type II and 15 Type III foci for a transformation frequency of  $5.64 \times 10^{-4}$  ( $p < 0.01$ ).

## CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data suggest that the test article T1866 (CMA-105) does induce morphological transformation in BALB/3T3 clone A31-1 cells.

TABLE 1

CYTOTOXIC EFFECTS OF T1866 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.53	100.0
MNNG 0.5 ug/ml	0.19	35.8
T1866 40 ug/ml	0.01	1.9
30 ug/ml	0.23	43.4
20 ug/ml	0.44	83.0

<sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORAMTION ASSAY

Study Number: T1866.302002

Study Director: D. PUTMAN, PH.D.

Initiation Date: DECEMBER 21, 1982

Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

TABLE 2

TRANSFORMATION POTENTIAL OF T1866 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	0/15	1/15	0.13
MNNG 0.5 ug/ml	3/14	15/14	5.64 <sup>c</sup>
T1866 40 ug/ml	3/14	2/14	14.29 <sup>d</sup>
30 ug/ml	5/15	4/15	1.16
20 ug/ml	0/15	1/15	0.15

<sup>a</sup> Cells seeded at 10<sup>4</sup> cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup> p<0.01, Modified Poisson Distribution

<sup>d</sup> p<0.05, Modified Poisson Distribution

CMA-106



DATES/PHASES

INSPECT ON 82/12/22 - 82/12/22, TO STUDY DIR 82/12/22, TO MGMT 82/12/28

PHASES: PROTOCOL REVIEW

INSPECT ON 83/03/14 - 83/03/14, TO STUDY DIR 83/03/14, TO MGMT 83/03/15

PHASES: SCORING TRANSFORMATION ASSAY PLATES

INSPECT ON 83/06/08 - 83/06/08, TO STUDY DIR 83/06/08, TO MGMT 83/10/27

PHASES: DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Nona S. Katten

Quality Assurance  
RA/QA Department

9/25/84  
Date

## RESULTS AND DISCUSSION

The test article CMA-106 was received by Microbiological Associates on 11-24-82 and was assigned the code number T1854. Storage requirements and expiration date were not specified. The test article was stored at room temperature. The test article was dissolved in acetone (CAS 67-64-1), lots 132200 and 110844, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. At the time of testing, T1854 was characterized as a clear viscous liquid.

T1854 was tested in the absence of an exogenous metabolic activation system at doses of 32, 24 and 16 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 32, 24 and 16 ug/ml in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 15%, 46% and 95%, respectively. The activity of T1854 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic system is presented in Table 2. One spontaneous Type III focus was observed in the solvent control for a background transformation frequency of  $0.17 \times 10^{-4}$ . One Type III focus was observed at 32 ug/ml, two Type III foci were observed at 24 ug/ml, and one Type II and three Type III foci were observed at 16 ug/ml. The transformation frequencies of the test article-treated groups were not statistically increased ( $p > 0.05$ ) when compared to that of the solvent control. MNNG induced 20 Type II and 21 Type III foci for a transformation frequency of  $35.0 \times 10^{-4}$  ( $p < 0.01$ ).

T1854 was tested in the presence of an exogenous metabolic activation system at doses of 20, 18, 16 and 14 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with Dulbecco's PBS. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 2 hour treatment of BALB/3T3 clone A31-1 cells in suspension culture with 20, 18, 16 and 14 ug/ml in the presence of an S-9 activation system are presented in Table 3. Relative to the acetone control, the cell survival was 13%, 19%, 47% and 91%, respectively. The activity of T1854 in the BALB/3T3 transformation system after a 2 hour treatment, in suspension, in the presence of an S-9 metabolic activation system is presented in Table 4. Two Type II and one Type III foci were observed in the acetone control for a background transformation frequency of  $0.24 \times 10^{-4}$ . Two Type II and five Type III foci were observed at 20 ug/ml, three Type II and six Type III foci

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-106)

Test Article Lot No.: Not Provided

MA Study No.: T1854.302002

Test Article Description: Clear Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 11/24/82

Initiation Date: 12/06/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor:

CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility:

MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director:

Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician:

John F. McCarvill 9/24/84  
John McCarvill, B.S. Date

TABLE 1

CYTOTOXIC EFFECTS OF T1854 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.41	100.0
MNNG 0.5 ug/ml	0.04	9.8
T1854 32 ug/ml	0.06	14.6
24 ug/ml	0.19	46.3
16 ug/ml	0.39	95.1

<sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

were observed at 18 ug/ml, four Type III foci were observed at 16 ug/ml, and two Type II and one Type III foci were observed at 14 ug/ml. The transformation frequencies of  $8.93 \times 10^{-4}$  at 20 ug/ml and  $7.14 \times 10^{-4}$  at 18 ug/ml were statistically increased ( $p < 0.01$ ) when compared to that of the solvent control. BaP induced five Type II and 14 Type III foci for a transformation frequency of  $5.83 \times 10^{-4}$  ( $p < 0.01$ ).

### CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicate that T1854 (CMA-106) induces morphological transformation of BALB/3T3 clone A31-1 cells but only when tested in the presence of an exogenous source of metabolic activation.

TABLE 3

CYTOTOXIC EFFECTS OF T1854 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE PRESENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.32	100.0
BaP 12.5 ug/ml	0.16	50.0
T1854 20 ug/ml	0.04	12.5
18 ug/ml	0.06	18.8
16 ug/ml	0.15	46.9
14 ug/ml	0.29	90.6

<sup>a</sup> Cells were treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml treatment medium containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of 250 cells/60 mm dish. After 7-10 days incubation, the cells were fixed, stained and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

TABLE 2

TRANSFORMATION POTENTIAL OF T1854 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	0/14	1/14	0.17
MNNG 0.5 ug/ml	20/15	21/15	35.00 <sup>c</sup>
T1854 32 ug/ml	0/15	1/15	1.11
24 ug/ml	0/14	2/14	0.75
16 ug/ml	1/14	3/14	0.55

<sup>a</sup> Cells seeded at 10<sup>4</sup> cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup> p<0.01, Modified Poisson Distribution

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY

Study Number: T1854.302002

Study Director: D. PUTMAN, PH.D.

Initiation Date: DECEMBER 6, 1982

( Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.



TABLE 4

TRANSFORMATION POTENTIAL OF T1854 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	2/13	1/13	0.24
BaP 12.5 ug/ml	5/15	14/15	5.83 <sup>c</sup>
T1854			
20 ug/ml	2/14	5/14	8.93 <sup>c</sup>
18 ug/ml	3/14	6/14	7.14 <sup>c</sup>
16 ug/ml	0/15	4/15	1.78
14 ug/ml	2/14	1/14	0.25

<sup>a</sup> Cells were treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml treatment medium containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of  $10^4$  cells/60 mm dish. After 4-6 weeks incubation, the cells were fixed, stained and scored for morphological transformation.

<sup>b</sup> Transformation frequency was reported as the number of Type III foci per surviving cell.

<sup>c</sup>  $p < 0.01$ , Modified Poisson Distribution

CMA-107

DATES/PHASES

( INSPECT ON 82/12/06 - 82/12/06, TO STUDY DIR 82/12/06, TO MGMT 82/12/06

PHASES: PROTOCOL REVIEW

INSPECT ON 83/01/17 - 83/01/17, TO STUDY DIR 83/01/17, TO MGMT 83/01/13

PHASES: REFEEDING CULTURES

INSPECT ON 83/04/20 - 83/04/20, TO STUDY DIR 83/04/20, TO MGMT 83/04/22

PHASES: TREATMENT OF TEST SYSTEM WITH TEST AND CONTROL ARTICLES

INSPECT ON 83/06/10 - 83/06/10, TO STUDY DIR 83/06/13, TO MGMT 83/10/27

PHASES: DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

(  
  
This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Mona S. Karter 9/25/84  
Quality Assurance Date  
RA/QA Department

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-107)

Test Article Lot No.: Not Provided

MA Study No.: T1857.302002

Test Article Description: Amber Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 11/24/82

Initiation Date: 12/06/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor: CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director: Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician: John T. McCarvill 9-24-84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article CMA-107 was received by Microbiological Associates on 11-24-82 and was assigned the code number T1857. Storage requirements and expiration date were not specified. The test article was stored at room temperature. The test article was dissolved in acetone (CAS 67-64-1), lot 132200, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. At the time of testing, T1857 was characterized as an amber viscous liquid.

T1857 was tested in the absence of an exogenous metabolic activation system at doses of 36, 27 and 18 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 36, 27 and 18 ug/ml in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 5%, 49% and 89% at 36, 27 and 18 ug/ml, respectively. The activity of T1857 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 2. No spontaneous foci were observed in the solvent control for a background transformation frequency of  $<0.18 \times 10^{-4}$ . One Type II and three Type III foci were observed at 36 ug/ml, one Type II and two Type III foci were observed at 27 ug/ml, and one Type III focus was observed at 18 ug/ml. The transformation frequency of  $10 \times 10^{-4}$  was statistically increased ( $p < 0.01$ ) when compared to that of the solvent control. MNNG induced 16 Type II and 27 Type III foci for a transformation frequency of  $25.71 \times 10^{-4}$  ( $p < 0.01$ ).

## CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicates that T1857 (CMA-107) induces a statistically significant transformation frequency at extremely toxic doses and should therefore be considered suspect in the BALB/3T3 transformation assay.

TABLE 1

CYTOTOXIC EFFECTS OF T1857 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

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Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.37	100.0
MNNG 0.5 ug/ml	0.07	18.9
T1857 36 ug/ml	0.02	5.4
27 ug/ml	0.18	48.6
18 ug/ml	0.33	89.2

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- <sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.
- <sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.
- <sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

TABLE 2

TRANSFORMATION POTENTIAL OF T1857 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	0/15	0/15	<0.18
MNNG 0.5 ug/ml	16/15	27/15	25.71 <sup>c</sup>
T1857 36 ug/ml	1/15	3/15	10.00 <sup>c</sup>
27 ug/ml	1/15	2/15	0.74
18 ug/ml	0/13	1/13	0.23

<sup>a</sup> Cells seeded at 10<sup>4</sup> cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup> p<0.01, Modified Poisson Distribution

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY

Study Number: T1857.302002

Study Director: D. PUTMAN, PH.D.

Initiation Date: DECEMBER 6, 1982

Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.



DATES/PHASES

INSPECT ON 82/12/06 - 82/12/06, TO STUDY DIR 82/12/06, TO MGMT 82/12/06

PHASES: PROTOCOL REVIEW

INSPECT ON 83/01/13 - 83/01/13, TO STUDY DIR 83/01/13, TO MGMT 83/01/21

PHASES: DILUTION OF TEST ARTICLE AND CONTROL ARTICLE

INSPECT ON 83/06/13 - 83/06/13, TO STUDY DIR 83/06/13, TO MGMT 83/10/27

PHASES: DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Nona S. Karter

Quality Assurance  
RA/QA Department

9/25/84

Date

CMA-108

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-108)

Test Article Lot No.: Not Provided

MA Study No.: T1852.302002

Test Article Description: Light Brown Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 11/24/82

Initiation Date: 11/29/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor: CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director: Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician: John J. McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article CMA-108 was received by Microbiological Associates on 11-24-82 and was assigned the code number T1852. Storage requirements and expiration date were not specified. The test article was stored at room temperature. The test article was dissolved in acetone (CAS 67-64-1), lots 132200, 110844 and 246618, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. At the time of testing, T1852 was characterized as a light brown viscous liquid.

T1852 was tested in the absence of an exogenous metabolic activation system at doses of 26, 18 and 10 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 26, 18 and 10 ug/ml in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 56%, 80% and 95% at 26, 18 and 10 ug/ml, respectively. The activity of T1852 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 2. One spontaneous Type III focus was observed in the solvent control for a background transformation frequency of  $0.12 \times 10^{-4}$ . One Type III focus was observed at 26 and 10 ug/ml. MNNG induced six Type II and seventeen Type III foci for a transformation frequency of  $28.3 \times 10^{-4}$  ( $p < 0.01$ , Modified Poisson Distribution).

T1852 was tested in the presence of an S-9 activation system at doses of 12, 11, 10 and 9 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with PBS. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 2 hour treatment of BALB/3T3 clone A31-1 cells with 12, 11, 10 and 9 ug/ml in the presence of an exogenous metabolic activation system are presented in Table 3. Relative to the acetone control, the cell survival was approximately 0%, <1%, 15% and 88% at 12, 11, 10 and 9 ug/ml, respectively. The activity of T1852 in the transformation system after a 2 hour treatment in suspension in the presence of S-9 activation system is presented in Table 4. One Type III focus was observed in the solvent control for a background transformation frequency of  $0.17 \times 10^{-4}$ . Seven Type II and six Type III foci were observed at 11 ug/ml, one Type II and one Type III foci were observed at 10 ug/ml, and one Type III focus was observed at 9 ug/ml. The induced transformation frequency was not calculated at 11 ug/ml due to the extreme level of toxicity. BaP induced one Type II

and eight Type III foci for a transformation frequency of  $1.52 \times 10^{-4}$  ( $p < 0.05$ , Modified Poisson Distribution).

### CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicate that the test article T1852 (CMA-108) does induce morphological transformation in BALB/3T3 clone A31-1 cells in the presence of an exogenous source of metabolic activation but only at levels of extreme toxicity. Since activity was found at a single dose with survival of  $< 1\%$ , the chemical should only be considered suspect in the BALB/3T3 transformation assay.

TABLE 1

CYTOTOXIC EFFECTS OF T1852 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.55	100.0
MNNG 0.5 ug/ml	0.04	7.3
T1852 26 ug/ml	0.31	56.4
18 ug/ml	0.44	80.0
10 ug/ml	0.52	94.5

<sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

TABLE 2

TRANSFORMATION POTENTIAL OF T1852 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	0/15	1/15	0.12
MNNG 0.5 ug/ml	6/15	17/15	28.33
T1852 26 ug/ml	0/15	1/15	0.22
18 ug/ml	0/15	0/15	<0.15
10 ug/ml	0/15	1/15	0.13

a Cells seeded at 10<sup>4</sup> cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

b Transformation frequency is reported as the number of Type III foci per surviving cell.

TABLE 3

CYTOTOXIC EFFECTS OF T1852 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE PRESENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.40	100.0
BaP 12.5 ug/ml	0.35	87.5
T1852 12 ug/ml	0.00	0.0
11 ug/ml	<0.01	<1.0
10 ug/ml	0.06	15.0
9 ug/ml	0.35	87.5

<sup>a</sup> Cells were treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml treatment medium containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of 250 cells/60 mm dish. After 7-10 days incubation, the cells were fixed, stained and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.



TABLE 4

TRANSFORMATION POTENTIAL OF T1852 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	0/15	1/15	0.17
BaP 12.5 ug/ml	1/15	8/15	1.52 <sup>c</sup>
T1852 12 ug/ml	- TOXIC -		
11 ug/ml	7/15	6/15	NC <sup>d</sup>
10 ug/ml	1/15	1/15	1.11
9 ug/ml	0/15	1/15	0.19

<sup>a</sup> Cells were treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml treatment medium containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of  $10^4$  cells/60 mm dish. After 4-6 weeks incubation, the cells were fixed, stained and scored for morphological transformation.

<sup>b</sup> Transformation frequency was reported as the number of Type III foci per surviving cell.

<sup>c</sup>  $p < 0.05$ , Modified Poisson Distribution.

<sup>d</sup> NC, not calculated due to extreme level of toxicity.

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY

Study Number: T1852.302002

Study Director: D. PUTMAN, PH.D.

Initiation Date: NOVEMBER 29, 1982

( Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

INSPECT ON 82/11/29 - 82/11/29, TO STUDY DIR 82/11/29, TO MGMT 82/11/29

PHASES: PROTOCOL REVIEW

INSPECT ON 83/02/04 - 83/02/04, TO STUDY DIR 83/02/04, TO MGMT 83/02/07

PHASES: SCORING TRANSFORMATION PLATES

INSPECT ON 83/08/05 - 83/08/05, TO STUDY DIR 83/08/05, TO MGMT 83/08/29

PHASES: REFEEDING OF CULTURES

INSPECT ON 83/10/07 - 83/10/11, TO STUDY DIR 83/10/11, TO MGMT 83/10/27

PHASES: DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Mona S. Harten 9/25/84  
Quality Assurance Date  
RA/QA Department

CMA-109

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-109)

Test Article Lot No.: Not Provided

MA Study No.: T1855.302002

Test Article Description: Amber Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 11/24/82

Initiation Date: 12/20/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor: CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director: Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician: John McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article CMA-109 was received by Microbiological Associates on 11-24-82 and was assigned the code number T1855. Storage requirements and expiration date were not specified. The test article was noted to have leaked in transit. The test article was stored at room temperature. The test article was dissolved in acetone (CAS 67-64-1), lots 132200 and 110844, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. At the time of testing, T1855 was characterized as an amber viscous liquid.

T1855 was tested in the absence of an exogenous metabolic activation system at doses of 60, 45 and 30 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 60, 45 and 30 ug/ml in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 1%, 31% and 67%, respectively. The activity of T1855 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 2. One spontaneous Type III focus was observed in the solvent control for a background transformation frequency of  $0.15 \times 10^{-4}$ . One Type II focus was observed at 60 ug/ml, one Type II and four Type III foci were observed at 45 ug/ml, and one Type II and three Type III foci were observed at 30 ug/ml. The transformation frequencies of the test article-treated groups were not statistically increased ( $p > 0.05$ ) when compared to that of the solvent control. MNNG induced 21 Type II and 26 Type III foci for a transformation frequency of  $86.67 \times 10^{-4}$  ( $p < 0.01$ ).

## CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicate that T1855 (CMA-109) induces a substantial although not statistically significant increase in transformation frequency at moderate to non-toxic dose levels and should be considered suspect in the BALB/3T3 transformation assay.

TABLE 1

CYTOTOXIC EFFECTS OF T1855 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.51	100.0
MNNG 0.5 ug/ml	0.02	3.9
T1855 60 ug/ml	0.003	0.6
45 ug/ml	0.16	31.4
30 ug/ml	0.34	66.7

<sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

TABLE 2

TRANSFORMATION POTENTIAL OF T1855 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> ( $\times 10^{-4}$ )
	Type II	Type III	
Acetone 2ul/ml	0/13	1/13	0.15
MNNG 0.5 ug/ml	21/15	26/15	86.67 <sup>c</sup>
T1855 60 ug/ml	1/15	0/15	<22.22
45 ug/ml	1/15	4/15	1.67
30 ug/ml	1/15	3/15	0.59

<sup>a</sup> Cells seeded at  $10^4$  cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup>  $p < 0.01$  Modified Poisson Distribution



QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY

Study Number: T1855.302002

Study Director: D. PUTMAN, PH.D.

Initiation Date: DECEMBER 20, 1982

( Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

( INSPECT ON 82/12/22 - 82/12/22, TO STUDY DIR 82/12/22, TO MGMT 82/12/22

PHASES: PROTOCOL REVIEW

INSPECT ON 83/02/08 - 83/02/08, TO STUDY DIR 83/02/08, TO MGMT 83/02/10

PHASES: DILUTION OF TEST ARTICLE

INSPECT ON 83/06/09 - 83/06/09, TO STUDY DIR 83/06/10, TO MGMT 83/10/27

PHASES : DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

( This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Mona S. Harten

Quality Assurance

RA/QA Department

9/25/84

Date

CMA-110

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-110)

Test Article Lot No.: Not Provided

MA Study No.: T1851.302002

Test Article Description: Dark Amber Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 11/24/82

Initiation Date: 12/07/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor: CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director:

Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician:

John T. McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article CMA-110 was received by Microbiological Associates on 11-24-82 and was assigned the code number T1851. Storage requirements and expiration date were not specified. The test article was stored at room temperature. The test article was dissolved in acetone (CAS 67-64-1), lots 132200 and 110844, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. At the time of testing, T1851 was characterized as a dark amber viscous liquid.

T1851 was tested in the absence of an exogenous metabolic activation system at doses of 26, 18 and 12 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 26, 18 and 12 ug/ml in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 56%, 78% and 98% at 26, 18 and 12 ug/ml, respectively. The activity of T1851 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 2. One spontaneous Type III focus was observed in the solvent control for a background transformation frequency of  $0.17 \times 10^{-4}$ . Two Type III foci were observed at 26 ug/ml, and one Type III focus was observed at 18 and 12 ug/ml. MNNG induced 20 Type II and 21 Type III foci for a transformation frequency of  $35.0 \times 10^{-4}$  ( $p < 0.01$ , Modified Poisson Distribution).

T1851 was tested in the presence of an S-9 activation system at doses of 14, 12, 10 and 8 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with PBS. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 2 hour treatment of BALB/3T3 clone A31-1 cells with 14, 12, 10 and 8 ug/ml in the presence of an exogenous metabolic activation system are presented in Table 3. Relative to the acetone control, the cell survival was approximately 70%, 85%, 89% and 83% at 14, 12, 10 and 8 ug/ml, respectively. The activity of T1851 in the transformation system after a 2 hour treatment in suspension in the presence of an S-9 activation system is presented in Table 4. One Type II and one Type III foci were observed in the solvent control for a background transformation frequency of  $0.16 \times 10^{-4}$ . Two Type II and seven Type III foci were observed at 14 ug/ml, two Type II and four Type III foci were observed at 12 ug/ml, three Type III foci were observed at 10 ug/ml and two Type III foci were observed at 8 ug/ml. The

induced transformation frequency of  $1.46 \times 10^{-4}$  at 14 ug/ml was statistically increased relative to the acetone control ( $p < 0.05$ , Modified Poisson Distribution). BaP induced seven Type II and seven Type III foci for a transformation frequency of  $2.27 \times 10^{-4}$  ( $p < 0.01$ , Modified Poisson Distribution).

### CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicate that the test article T1851 (CMA-110) does induce morphological transformation in BALB/3T3 clone A31-1 cells in the presence of an exogenous source of metabolic activation.

TABLE 1

CYTOTOXIC EFFECTS OF T1851 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.41	100.0
MNNG 0.5 ug/ml	0.04	9.8
T1851 26 ug/ml	0.23	56.1
18 ug/ml	0.32	78.0
12 ug/ml	0.40	97.6

<sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

TABLE 2

TRANSFORMATION POTENTIAL OF T1851 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	0/14	1/14	0.17
MNNG 0.5 ug/ml	20/15	21/15	35.00 <sup>c</sup>
T1851 26 ug/ml	0/15	2/15	0.58
18 ug/ml	0/15	1/15	0.21
12 ug/ml	0/15	1/15	0.17

<sup>a</sup> Cells seeded at 10<sup>4</sup> cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup> p<0.01, Modified Poisson Distribution.



TABLE 3

CYTOTOXIC EFFECTS OF T1851 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE PRESENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.46	100.0
BaP 12.5 ug/ml	0.22	47.8
T1851 14 ug/ml	0.32	69.6
12 ug/ml	0.39	84.8
10 ug/ml	0.41	89.1
8 ug/ml	0.38	82.6

<sup>a</sup> Cells were treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml treatment medium containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of 250 cells/60 mm dish. After 7-10 days incubation, the cells were fixed, stained and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY

Study Number: T1851.302002

Study Director: D. PUTMAN, PHD.

Initiation Date: DECEMBER 7, 1982

( Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

TABLE 4

TRANSFORMATION POTENTIAL OF T1851 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	1/14	1/14	0.16
BaP 12.5 ug/ml	7/14	7/14	2.27 <sup>c</sup>
T1851 14 ug/ml	2/15	7/15	1.46 <sup>d</sup>
12 ug/ml	2/15	4/15	0.68
10 ug/ml	0/14	3/14	0.52
8 ug/ml	0/15	2/15	0.35

<sup>a</sup> Cells were treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml treatment medium containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of  $10^4$  cells/60 mm dish. After 4-6 weeks incubation, the cells were fixed, stained and scored for morphological transformation.

<sup>b</sup> Transformation frequency was reported as the number of type III foci per surviving cell.

<sup>c</sup>  $p < 0.01$ , Modified Poisson Distribution.

<sup>d</sup>  $p < 0.05$ , Modified Poisson Distribution.

CMA-111

DATES/PHASES

INSPECT ON 82/12/06 - 32/12/06, TO STUDY DIR 82/12/06, TO MGMT 82/12/06

PHASES: PROTOCOL REVIEW

INSPECT ON 83/01/11 - 33/01/11, TO STUDY DIR 83/01/11, TO MGMT 83/01/14

PHASES: DILUTION OF TEST ARTICLE AND CONTROL ARTICLE

INSPECT ON 83/05/24 - 33/05/24, TO STUDY DIR 83/05/24, TO MGMT 83/06/01

PHASES: REFEEDING OF CULTURES

INSPECT ON 83/10/11 - 33/10/11, TO STUDY DIR 83/10/11, TO MGMT 83/10/27

PHASES: DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

*Ronald S. Kerten*      9/25/84  
Quality Assurance      Date  
RA/QA Department

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-111)

Test Article Lot No.: Not Provided

MA Study No.: T1867.302002

Test Article Description: Dark Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 12/10/82

Initiation Date: 12/21/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor: CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director: Donald L. Putman 9-28-84  
Donald L. Putman, Ph.D. Date

Lead Technician: John T. McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article CMA-111 was received by Microbiological Associates on 12-10-82 and was assigned the code number T1867. Storage requirements and expiration date were not specified. The test article was stored at room temperature. The test article was dissolved in acetone (CAS 67-64-1), lot 110844, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. AT the time of testing, T1867 was characterized as a dark viscous liquid.

T1867 was tested in the absence of an exogenous metabolic activation system at doses of 40, 30 and 20 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2x) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 40, 30 and 20 ug/ml in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 12%, 43% and 80% at 40, 30 and 20 ug/ml, respectively. The activity of T1867 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 2. One Type III focus was observed in the solvent control for a background transformation frequency of  $0.15 \times 10^{-4}$ . Two Type II and four Type III foci were observed at 40 ug/ml, one Type II and six Type III foci were observed at 30 ug/ml, and one Type III focus was observed at 20 ug/ml. The transformation frequencies of  $4.44 \times 10^{-4}$  at 40 ug/ml and  $1.95 \times 10^{-4}$  at 30 ug/ml were statistically increased ( $p < 0.01$ ) when compared to that of the solvent control. MNNG induced 21 Type II and 26 Type III foci for a transformation frequency of  $86.67 \times 10^{-4}$  ( $p < 0.01$ ).

## CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicate that the test article T1867 (CMA-111) does induce morphological transformation in BALB/3T3 clone A31-1 cells.

TABLE 1

CYTOTOXIC EFFECTS OF T1867 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.51	100.0
MNNG 0.5 ug/ml	0.02	3.9
T1867 40 ug/ml	0.06	11.8
30 ug/ml	0.22	43.1
20 ug/ml	0.41	80.4

- <sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.
- <sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.
- <sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.



TABLE 2

TRANSFORMATION POTENTIAL OF T1867 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	0/13	1/13	0.15
MNNG 0.5 ug/ml	21/15	26/15	86.67 <sup>c</sup>
T1867 40 ug/ml	2/15	4/15	4.44 <sup>c</sup>
30 ug/ml	1/14	6/14	1.95 <sup>c</sup>
20 ug/ml	0/13	1/13	0.19

<sup>a</sup> Cells seeded at 10<sup>4</sup> cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup> p<0.01, Modified Poisson Distribution

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY.

Study Number: T1867.302002

Study Director: D. PUTMAN, PH.D.

Initiation Date: DECEMBER 21, 1982

Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

( INSPECT ON 82/12/22 - 82/12/22, TO STUDY DIR 82/12/28, TO MGMT 82/12/28

PHASES: PROTOCOL REVIEW

INSPECT ON 83/02/25 - 83/02/25, TO STUDY DIR 83/02/25, TO MGMT 83/03/15

PHASES: REFEEDING CULTURES

INSPECT ON 83/06/08 - 83/06/09, TO STUDY DIR 83/06/09, TO MGMT 83/10/27

PHASES: DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

(  
  
This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

*Nona S. Harten* 9/25/84  
Quality Assurance Date  
RA/QA Department

CMA-112

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc dialkyldithiophosphate (Code CMA-112)

Test Article Lot No.: Not Provided

MA Study No.: T1853.302002

Test Article Description: Clear Amber, Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 11/24/82

Initiation Date: 11/29/82

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor:

CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility:

MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director:

Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician:

John T. McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article CMA-112 was received by Microbiological Associates on 11-24-82 and was assigned the code number T1853. Storage requirements and expiration date were not specified. The test was stored at room temperature. The test article was dissolved in acetone (CAS-67-64-1), lot 132200, obtained from J.T. Baker Chemical Company, Phillipsburg, N.J. At the time of testing, T1853 was characterized as a clear amber, viscous liquid.

T1853 was tested in the absence of an exogenous metabolic activation system at doses of 20, 17 and 14 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in acetone and then diluted to the appropriate (2X) concentration with complete EMEM. The solvent control dishes were treated with acetone at a final concentration of 2 ul/ml. The cytotoxic effects of a 24 hour treatment of BALB/3T3 clone A31-1 cells with 20, 17 and 14 ug/ml in the absence of an exogenous metabolic activation system are presented in Table 1. Relative to the acetone control, the cell survival was approximately 57%, 73% and 97% at 20, 17 and 14 ug/ml, respectively. The activity of T1853 in the BALB/3T3 transformation system after a 24 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 2. No spontaneous foci were observed in the solvent control for a background transformation frequency of  $<0.18 \times 10^{-4}$ . Seven Type III foci were observed at 20 ug/ml, and two Type III foci were observed at 14 ug/ml. The transformation frequency of  $2.22 \times 10^{-4}$  at 20 ug/ml was statistically increased ( $p < 0.01$ ) when compared to that of the solvent control. MNNG induced 16 Type II and 27 Type III foci for a transformation frequency of  $25.71 \times 10^{-4}$  ( $p < 0.01$ ).

## CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicates that the test article T1853 (CMA-112) does induce morphological transformation in BALB/3T3 clone A31-1 cells.

TABLE 1

CYTOTOXIC EFFECTS OF T1853 IN BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE ABSENCE OF EXOGENOUS  
METABOLIC ACTIVATION

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Acetone 2 ul/ml	0.37	100.0
MNNG 0.5 ug/ml	0.07	18.9
T1853 20 ug/ml	0.21	56.8
17 ug/ml	0.27	73.0
14 ug/ml	0.36	97.3

<sup>a</sup> Cells seeded at 250 cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 7-10 days. Cells were fixed, stained with Giemsa, and scored for colony formation.

<sup>b</sup> Cloning efficiency was reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency was reported as the number of colonies per treatment condition relative (normalized) to 100% survival in the solvent control.

TABLE 2

TRANSFORMATION POTENTIAL OF T1853 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Acetone 2 ul/ml	0/15	0/15	<0.18
MNNG 0.5 ug/ml	16/15	27/15	25.71 <sup>c</sup>
T1853 20 ug/ml	0/15	7/15	2.22 <sup>c</sup>
17 ug/ml	1/14	0/14	<0.26
14 ug/ml	0/15	2/15	0.37

<sup>a</sup> Cells seeded at 10<sup>4</sup> cells/dish were treated the following day for 24 hours at 36°C. The treatment medium was washed off and the cells were cultured in complete EMEM for 4-6 weeks. Cells were fixed, stained with Giemsa, and scored for morphologically transformed foci.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell.

<sup>c</sup> p<0.01 Modified Poisson Distribution



QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMTION ASSAY

Study Number: T1853.302002

Study Director: D. PUTMAN, PH.D.

Initiation Date: NOVEMBER 29, 1982

( Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

ZINC CHLORIDE

DATES/PHASES

( INSPECT ON 82/11/29 - 82/11/29, TO STUDY DIR 82/11/29, TO MGMT 82/11/29

PHASES: PROTOCOL REVIEW

INSPECT ON 83/01/18 - 83/01/18, TO STUDY DIR 83/01/18, TO MGMT 83/01/21

PHASES: DILUTION OF TEST ARTICLE AND CONTROL ARTICLE

INSPECT ON 83/01/18 - 83/01/18, TO STUDY DIR 83/01/18, TO MGMT 83/01/21

PHASES: WEIGHING OF TEST ARTICLE


INSPECT ON 83/06/08 - 83/06/09, TO STUDY DIR 83/06/09, TO MGMT 83/10/27

PHASES: DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

(  
  
This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

  
\_\_\_\_\_  
Quality Assurance                      9/25/84  
RA/QA Department                      Date

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Zinc chloride

Test Article Lot No.: KTJY

MA Study No.: T2255.302

Test Article Description: White granules

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 12/12/83

Initiation Date: 4/3/84

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor: CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director: Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician: John T. McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article was received by Microbiological Associates on December 12, 1983 and was assigned the code number T2255. Storage requirements and expiration date were not specified. Upon receipt, the test article was described as white granules and was stored in the dark at room temperature. The test article was dissolved in distilled water (CAS 7732-18-5), lot 13P880, obtained from Gibco Laboratories, Grand Island, NY, and dosing solutions were prepared in PBS immediately prior to use. At the time of testing, T2255 was described as white granules.

T2255 was tested in the presence of an exogenous metabolic activation system at doses of 800, 600, 400 and 200 ug/ml which were selected following a preliminary dose range-finding clonal toxicity assay. Dosing solutions were prepared in distilled water and then diluted to the appropriate (2X) concentration in PBS. The solvent control dishes were treated with distilled water at a final concentration of 2 ul/ml.

The cytotoxic effects of a 2 hour treatment of BALB/3T3 clone A31-1 cells with 800, 600, 400 and 200 ug/ml in the presence of a rat liver S-9 activation system are presented in Table 1. Relative to the solvent control, the cell survival was approximately 1%, 2%, 17% and 83% at 800, 600, 400 and 200 ug/ml, respectively. The activity of T2255 in the transformation assay under the same treatment conditions are presented in Table 2. Two Type II but no spontaneous Type III foci were observed in the solvent control for a background transformation frequency of  $<0.22 \times 10^{-4}$ . Treatments at 800 and 600 ug/ml were extremely toxic and the cells in the transformation dishes failed to grow. One Type II and 3 Type III foci were observed at 400 ug/ml, and two Type II and 5 Type III foci were observed at 200 ug/ml. The induced transformation frequencies of  $4.00 \times 10^{-4}$  and  $1.43 \times 10^{-4}$  were statistically increased above the solvent control ( $p < 0.01$  and  $p < 0.05$ , respectively, Modified Poisson Distribution). BaP induced 7 Type II and 7 Type III foci for a transformation frequency of  $2.17 \times 10^{-4}$  ( $p < 0.01$ , Modified Poisson Distribution).

## CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the data indicate that the test article, Zinc chloride, does induce morphological transformation of BALB/3T3 clone A31-1 cells in the presence of rat liver S-9.

TABLE 1

**CYTOTOXIC EFFECTS OF T2255 TO BALB/3T3 CLONE A31-1  
CELLS EXPOSED IN THE PRESENCE OF EXOGENOUS  
METABOLIC ACTIVATION**

Treatment <sup>a</sup>	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
Water 4 ul/ml	0.30	100.0
BaP 12.5 ug/ml	0.23	76.7
T2255 800 ug/ml	0.003	1.0
600 ug/ml	0.007	2.3
400 ug/ml	0.05	16.7
200 ug/ml	0.25	83.3

- <sup>a</sup> Cells treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml PBS containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of 250 cells/60 mm dish. After 7-10 days incubation, the cells were fixed, stained and scored for colony formation.
- <sup>b</sup> Cloning efficiency is reported as the number of colonies per number of cells seeded.
- <sup>c</sup> Relative cloning efficiency is reported as the number of colonies per treatment condition relative to 100% survival in the solvent control.

TABLE 2

TRANSFORMATION POTENTIAL OF T2255 IN  
BALB/3T3 CLONE A31-1 CELLS EXPOSED IN THE  
PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment <sup>a</sup>	Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (x 10 <sup>-4</sup> )
	Type II	Type III	
Water			
4 ul/ml	2/15	0/15	0.22
BaP			
12.5 ug/ml	7/14	7/14	2.17**
T2255			
800 ug/ml	- TOXIC -		
600 ug/ml	- TOXIC -		
400 ug/ml	1/15	3/15	4.00**
200 ug/ml	2/14	5/14	1.43*

<sup>a</sup> Cells treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml PBS containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of  $10^4$  cells/60 mm dish. After 4-6 weeks incubation, the cells were fixed, stained and scored for morphological transformation.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ .

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY

Study Number: T2255.302

Study Director: D. PUTMAN, PH.D.

Initiation Date: APRIL 3, 1984

Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.



DATES/PHASES

INSPECT ON 84/03/20 - 84/03/20, TO STUDY DIR 84/03/20, TO MGMT 84/03/20

PHASES: PROTOCOL REVIEW

INSPECT ON 84/04/30 - 84/04/30, TO STUDY DIR 84/04/30, TO MGMT 84/05/01

PHASES: SCORING & RECORDING PARALLEL TOXICITY PLATES

INSPECT ON 84/06/07 - 84/06/07, TO STUDY DIR 84/06/07, TO MGMT 84/06/08

PHASES: DRAFT REPORT

INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24

PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

*Mona S. Karter*

Quality Assurance  
RA/QA Department

*9/25/84*  
Date

CALCIUM DIALKYLDITHIOPHOSPHATE

BALB/3T3 TRANSFORMATION ASSAY

Test Article I.D.: Calcium dialkyldithiophosphate

Test Article Lot No.: Not Provided

MA Study No.: T2253.302

Test Article Description: Light Green, Viscous Liquid

Storage Conditions: Room Temperature;  
Protect from Light

Date Sample Received: 12/12/83

Initiation Date: 3/30/84

Completion Date: 9/24/84

Report Date: 9/25/84

Sponsor: CHEMICAL MANUFACTURERS ASSOCIATION  
2501 M Street, N.W.  
Washington, D.C. 20037

Authorized Representative: Carol R. Stack, Ph.D.

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

Study Director: Donald L. Putman 9-24-84  
Donald L. Putman, Ph.D. Date

Lead Technician: John T. McCarvill 9/24/84  
John McCarvill, B.S. Date

## RESULTS AND DISCUSSION

The test article, calcium dialkyldiothiophosphate, was received at Microbiological Associates, Inc. on December 12, 1983 and was assigned the code number T2253. Strength, purity and stability data were not provided. No expiration date was provided. Upon receipt, the test article was described as a light green viscous liquid. At the time of use, the test article was diluted with acetone (CAS 67-64-1), lot 73025 and lot 735031, obtained from Fisher Chemical Company, Springfield, NJ. At the time of testing, T2253 was described as a light green, viscous liquid. The stability of the test article under the experimental conditions was not determined by Microbiological Associates, Inc.

Dose levels for the cell transformation assay were selected following a preliminary toxicity test based upon cloning efficiency after treatment relative to the solvent control (Table 1). BALB/3T3 cells were exposed to solvent alone or to seven concentrations of test article ranging from 1 ul/ml to 0.001 ul/ml in the presence of an S-9 reaction mixture. A repeat preliminary toxicity was conducted using a narrower range (0.03 - 0.01 ul/ml) over 5 dose levels. Based upon these findings, dose levels of 0.05, 0.04, 0.03 and 0.02 ul/ml were selected for further study.

The cytotoxic effects of a 2 hour treatment of BALB/3T3 clone A31-1 cells in the presence of an exogenous metabolic activation system is presented in Table 2. The first assay performed was invalidated due to a high spontaneous background rate in the vehicle control; therefore only the results from a repeat assay are reported. Relative to the solvent control, the cell survival was 87%, 92%, 96% and 90% at 0.05, 0.04, 0.03 and 0.02 ul/ml, respectively. The activity of T2253 in the transformation assay is presented in Table 3. No Type II or Type III foci were observed in the solvent control for a background frequency of  $<0.13 \times 10^{-4}$ . No foci were observed in the test-article treated groups. BaP induced 5 Type III foci for a transformation frequency of  $1.33 \times 10^{-4}$  ( $p < 0.01$ , Modified Poisson Distribution).

Since the treatments were relatively nontoxic in the transformation assay, the assay was repeated using 0.06, 0.05, 0.04 and 0.03 ul/ml. The cytotoxic effects of treatment in the second study are presented in Table 4. Relative to the solvent control, the cell survival was 39%, 68%, 83% and 83% at 0.06, 0.05, 0.04 and 0.03 ul/ml, respectively. The activity of T2253 in the cell transformation assay is presented in Table 5. No Type II or Type III foci were observed in the solvent control group for a background transformation frequency of  $<0.17 \times 10^{-4}$ . One Type II and 2 Type III foci were observed at 0.06 ul/ml, 3 Type II but no Type III foci were observed at 0.05 ul/ml, 2 Type II but no Type III foci were observed at 0.04 ul/ml, and 1 Type

( II and 9 Type III foci were observed at 0.03 ul/ml. None of the transformation frequencies in the test article-treated groups were statistically increased relative to the solvent control group ( $p > 0.05$ , Modified Poisson Distribution). BaP induced 9 Type II and 10 Type III foci for a transformation frequency of  $2.86 \times 10^{-4}$  ( $p < 0.01$ , Modified Poisson Distribution).

### CONCLUSIONS

The negative control and the positive control fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, calcium dialkyldithiophosphate does not induce a significant level of morphological transformation in BALB/3T3 clone A31-1 cells when tested in the presence of an S-9 activation system and, therefore, should be considered negative in this assay system.

TABLE 1  
CYTOTOXIC EFFECTS OF T2253 TO BALB/3T3 CLONE A31-1 CELLS  
EXPOSED IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Preliminary Toxicity Test

Treatment <sup>a</sup>	Number of Colonies			Total Colonies	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
	Plate 1	Plate 2	Plate 3			
INITIAL ASSAY						
Acetone 2 ul/ml	91	102	105	298	0.40	100
T2253 1.0 ul/ml	0	0	0	0	0	0
0.3 ul/ml	0	0	0	0	0	0
0.1 ul/ml	0	0	0	0	0	0
0.03 ul/ml	6	4	2	12	0.02	5
0.01 ul/ml	106	103	100	309	0.41	103
0.003 ul/ml	105	103	100	308	0.41	103
0.001 ul/ml	101	102	99	302	0.40	100
-----						
REPEAT ASSAY						
Acetone 2 ul/ml	107	107	100	314	0.42	100
T2253 0.030 ul/ml	54	59	62	175	0.23	55
0.025 ul/ml	52	52	52	156	0.21	50
0.020 ul/ml	92	99	88	279	0.37	88
0.015 ul/ml	100	92	98	290	0.39	93
0.010 ul/ml	104	107	103	314	0.42	100

<sup>a</sup> Cells treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml PBS containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of 250 cells/60 mm dish. After 7-10 days incubation, the cells were fixed, stained and scored for colony formation.

<sup>b</sup> Cloning efficiency is reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency is reported as the number of colonies per treatment condition relative to 100% survival in the solvent control.

TABLE 2  
CYTOTOXIC EFFECTS OF T2253 TO BALB/3T3 CLONE A31-1 CELLS  
EXPOSED IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Concurrent Toxicity Test for Assay 1

Treatment <sup>a</sup>	Number of Colonies			Total Colonies	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
	Plate 1	Plate 2	Plate 3			
Acetone 2 ul/ml	125	131	131	387	0.52	100
BaP 12.5 ug/ml	73	70	72	215	0.29	56
T2253 0.05 ul/ml	113	114	109	336	0.45	87
0.04 ul/ml	119	122	122	363	0.48	92
0.03 ul/ml	120	126	129	375	0.50	96
0.02 ul/ml	121	114	120	355	0.47	90

<sup>a</sup> Cells treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml PBS containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of 250 cells/60 mm dish. After 7-10 days incubation, the cells were fixed, stained and scored for colony formation.

<sup>b</sup> Cloning efficiency is reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency is reported as the number of colonies per treatment condition relative to 100% survival in the solvent control.

TABLE 3  
TRANSFORMATION POTENTIAL OF T2253 TO BALB/3T3 CLONE A31-1 CELLS  
EXPOSED IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Assay 1

Treatment <sup>a</sup>	Dishes with Foci/Total Dishes		Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (X 10 <sup>-4</sup> )
	Type II	Type III	Type II	Type III	
Acetone 2 ul/ml	0/15	0/15	0/15	0/15	<0.13
BaP 12.5 ug/ml	0/13	5/13	0/13	5/13	1.33**
T2253 0.05 ul/ml	0/15	0/15	0/15	0/15	<0.15
0.04 ul/ml	0/15	0/15	0/15	0/15	<0.14
0.03 ul/ml	0/15	0/15	0/15	0/15	<0.13
0.02 ul/ml	0/15	0/15	0/15	0/15	<0.14

<sup>a</sup> Cells treated for 2 hours at a density of  $2 \times 10^6$  cells/4 ml PBS containing 100 ul/ml S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of  $10^5$  cells/60 mm dish. After 4-6 weeks incubation, the cells were fixed, stained and scored for morphological transformation.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ .



TABLE 4  
CYTOTOXIC EFFECTS OF T2253 TO BALB/3T3 CLONE A31-1 CELLS  
EXPOSED IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Concurrent Toxicity Test for Assay 2

Treatment <sup>a</sup>	Number of Colonies			Total Colonies	Cloning Efficiency <sup>b</sup>	Relative Cloning Efficiency (%) <sup>c</sup>
	Plate 1	Plate 2	Plate 3			
Acetone 2 ul/ml	104	103	98	305	0.41	100
BaP 12.5 ug/ml	82	51	54	187	0.25	61
T2253 0.06 ul/ml	41	40	42	123	0.16	39
0.05 ul/ml	70	70	73	213	0.28	68
0.04 ul/ml	86	85	84	255	0.34	83
0.03 ul/ml	81	92	83	256	0.34	83

<sup>a</sup> Cells treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml PBS containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of 250 cells/60 mm dish. After 7-10 days incubation, the cells were fixed, stained and scored for colony formation.

<sup>b</sup> Cloning efficiency is reported as the number of colonies per number of cells seeded.

<sup>c</sup> Relative cloning efficiency is reported as the number of colonies per treatment condition relative to 100% survival in the solvent control.

TABLE 5  
TRANSFORMATION POTENTIAL OF T2253 TO BALB/3T3 CLONE A31-1 CELLS  
EXPOSED IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Assay 2

Treatment <sup>a</sup>	Dishes with Foci/Total Dishes		Total Foci/Total Dishes		Transformation Frequency <sup>b</sup> (X 10 <sup>-4</sup> )
	Type II	Type III	Type II	Type III	
Acetone 2 ul/ml	0/14	0/14	0/14	0/14	<0.17
BaP 12.5 ug/ml	7/14	6/14	9/14	10/14	2.86**
T2253 0.06 ul/ml	1/15	2/15	1/15	2/15	0.83
0.05 ul/ml	3/15	0/15	3/15	0/15	<0.24
0.04 ul/ml	2/15	0/15	2/15	0/15	<0.20
0.03 ul/ml	1/14	2/14	1/14	2/14	0.42

<sup>a</sup> Cells treated for 2 hours in suspension at a density of  $2 \times 10^6$  cells/4 ml PBS containing 100 ul S-9, 1.9 mM NADP, 1.4 mM NADH, 1.9 mM NADPH, 7.4 mM glucose-6-phosphate and the appropriate concentration of test article or control. The treatment medium was removed and the cells were cultured at a density of  $10^4$  cells/60 mm dish. After 4-6 weeks incubation, the cells were fixed, stained and scored for morphological transformation.

<sup>b</sup> Transformation frequency is reported as the number of Type III foci per surviving cell; \*\*,  $p \leq 0.01$ ; \*,  $p \leq 0.05$ .

QUALITY ASSURANCE STATEMENT

Study Title: BALB/3T3 TRANSFORMATION ASSAY

Study Number: T2253.302

Study Director: D. PUTMAN, PH.D.

Initiation Date: MARCH 30, 1984

Review Completed Date: SEPTEMBER 24, 1984

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATES/PHASES

( INSPECT ON 84/03/20 - 84/03/20, TO STUDY DIR 84/03/20, TO MGMT 84/03/20  
PHASES: PROTOCOL REVIEW

INSPECT ON 84/05/03 - 84/05/03, TO STUDY DIR 84/05/03, TO MGMT 84/05/03  
PHASES: DILUTION OF TEST ARTICLE & POSITIVE CONTROL

INSPECT ON 84/07/06 - 84/07/06, TO STUDY DIR 84/07/06, TO MGMT 84/07/09  
PHASES: REFEEDING OF TRANSFORMATION PLATES

INSPECT ON 84/07/27 - 84/07/27, TO STUDY DIR 84/07/27, TO MGMT 84/07/31  
PHASES: REFEEDING - 2ND WEEK

INSPECT ON 84/08/29 - 84/08/30, TO STUDY DIR 84/08/30, TO MGMT 84/08/30  
PHASES: DRAFT REPORT

( INSPECT ON 84/09/24 - 84/09/24, TO STUDY DIR 84/09/24, TO MGMT 84/09/24  
PHASES: DRAFT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Mona S. Karter  
Quality Assurance  
RA/QA Department

9/25/84  
Date

## REFERENCES

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